Effect of dietary vitamin A on Senegalese sole (*Solea senegalensis*) skeletogenesis and larval quality

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Abstract

The effects of different levels of vitamin A (VA) in Senegalese sole larval performance and development were evaluated by means of a dietary dose-response experiment using enriched *Artemia* metanauplii as a carrier of this micronutrient. Larvae were fed from 6 to 27 days post hatch (dph) with enriched *Artemia* containing graded levels of total VA (1.3, 2.1, 4.5 and 12.9 μg VA mg^{-1} DW). The content of VA in live prey directly affected its accumulation in larvae and early juveniles. Retinyl palmitate accumulated during larval ontogeny, whereas retinol showed the opposite trend, decreasing from hatching until 41 dph and then remaining constant until the end of the study.

In metamorphic larvae (10 and 15 dph), VA did not affect the number of thyroid follicles or the intensity of the immunoreactive staining of T_3 and T_4. However, at older stages of development (post-metamorphic larvae: 20, 30, 41 and 48 dph), VA decreased the number of thyroid follicles but increased their mean size and enhanced T_3 and T_4 immunoreactive staining. A dietary excess of VA did not affect either larval performance in terms of growth and survival or the maturation of the digestive system. However, the most remarkable impact of this morphogenetic nutrient was detected during skeletal morphogenesis. Dietary VA accelerated the intramembranous ossification of vertebral centrums, which led to the formation of a supranumerary haemal vertebra and a high incidence of fused and compressed vertebrae in fish fed 2.1, 4.5 and 12.9 mg VA mg^{-1} DW. In addition, VA also affected those structures from vertebrae and caudal fin formed by chondral ossification, leading to defects in their shape and fusions with adjacent skeletal elements. In particular, the caudal fin was the region most affected by the dietary treatments. In order of importance, the bones with more developmental anomalies were the modified neural and haemal spines, epural, hypurals and parahypural. The impact of systemic factors such as thyroidal hormones in skeletogenesis should not be neglected since present results revealed that an excess of dietary VA affected the levels of T_3 and T_4, which might have affected bone formation and remodelling, leading to skeletal deformities.

Key words: Senegalese sole; *Solea senegalensis*; larval quality; vitamin A; skeleton; thyroid hormones; deformities.

1. Introduction

Since the nineties, Senegalese sole (*Solea senegalensis* Kaup, 1858) has been considered a promising flatfish species for diversifying European marine aquaculture (Dinis et al., 1999). Recently, as profit margins for the two main cultured Southern European fish species, gilthead sea bream and European sea bass, have decreased due to their overproduction, interest has increased in Senegalese sole farming in Mediterranean and Southern Atlantic waters. Some of the advantages of culturing Senegalese sole include its high market price, the natural spawning of wild broodstocks held in captivity and mass production of offspring, the rapid development of eggs and larvae, and the high growth rate exhibited by juveniles (see review in Dinis et al., 1999). However, several bottlenecks compromise the intensive culture of this flatfish species, such as the reproduction of F1 broodstock (Anguis and Cañavate, 2005), pathological outbreaks (Zarza et al., 2003), and the production of juveniles in proper quantity and quality to satisfy market demands (high incidence of pigmentary disorders and skeletal deformities) (Soares et al., 2002; Gavaia et al., 2002).

Skeletal deformities and pigmentary disorders are important factors affecting flatfish production costs and determining the fish external morphology, appearance, growth, survival rate, and final market price (Takeuchi et al., 1998; Gavaia et al., 2002; Hamre et al., 2005). The development of these abnormalities is linked to a poorly understood relationship between nutritional, environmental, and genetic factors. Among them, larval nutrition at first feeding is one of the key parameters that affect skeletogenesis and pigmentation processes during early development. In this regard, several studies have shown that nutrients are responsible for the appearance of

skeletal deformities and pigmentation disorders when their level and/or form of supply in the diet are inappropriate or unbalanced (see review in Lall and Lewis-McCrea, 2007; Hamre et al., 2005). Several authors have indicated that colour abnormalities in Japanese flounder could be effectively reduced by feeding larvae with high doses of vitamin A (VA) (Estévez and Kanazawa, 1995; Dedi et al., 1997; Takeuchi et al., 1995; Haga et al., 2002; Tarui et al., 2006). However, larvae fed high levels of VA showed a high incidence of skeletal deformities (Estévez and Kanazawa, 1995; Dedi et al., 1997; Takeuchi et al., 1998; Martínez et al., 2007) due to the morphogenetic action of this nutrient, which is known to have teratogenic effects in vertebrates at inappropriate dietary levels (Ross et al., 2000). Thus, in a situation in which a given nutrient exerts positive and negative effects simultaneously on different quality parameters, it is very important to determine a safe level that assures a normal skeletal development (minimum incidence of skeletal deformities) while preventing pigmentary disorders (pseudoalbinism and/or ambicolouration). The rapid physiological changes that Senegalese sole larvae undergo throughout development, reaching a fully metamorphosed morphology at an age of 20 days at 20ºC (Fernández-Díaz et al., 2001), make this species of particular interest for studying the dietary effects of vitamin A on skeletogenesis and metamorphosis.

The objective of the present study was to evaluate the effect of graded levels of dietary VA administered to Senegalese sole larvae during the *Artemia* feeding phase on larval performance (growth, survival, maturation of the digestive function, and metamorphosis success) and quality (incidence and typology of skeletal deformities).

2. Materials and methods

2.1 Larval rearing and experimental diets

Newly hatched larvae of Senegalese sole were obtained from Stolt Sea Farm SA (Cambre, La Coruña, Spain) and shipped by road to IRTA facilities. After their

113 acclimation, larvae were distributed (initial density: 50 larvae l^{-1}) in 12 cylindrical tanks
114 (100 l) connected to a recirculation unit (Carbó et al., 2003). Water conditions were as
115 follows: 18 ± 1 ºC, 35 ppt salinity, pH between 7.8-8.2, and daily exchange of water
116 (20%) in the recirculation system with gentle aeration and oxygenation (> 4 mg l^{-1}).
117 Photoperiod was 12L:12D, and light intensity was 500 lux at water surface.
118 Figure 1 shows the feeding protocol for *Solea senegalensis* used in the present
119 study. In detail, larvae were fed from day 3 post hatch (dph) to 10 dph with rotifers
120 (*Brachionus plicatilis*) enriched with Easy Selco™ (ES, INVE, Belgium) following
121 manufacturer's instructions. Rotifer density was 10 rotifers ml^{-1} from 3 to 4 dph and
122 gradually reduced to 5 rotifers ml^{-1} at 10 dph. Rotifer density was adjusted twice a day
123 in order to assure the optimal prey density. Enriched *Artemia* metanauplii (EG, INVE,
124 Belgium) were offered to larvae from 6 to 37 dph at increasing densities from 0.5 to 12
125 metanauplii ml^{-1}. *Artemia* metanauplii density was adjusted four times per day (at 9, 12,
126 15 and 18 h) to assure the optimal prey density and nutritional VA value; adjustments
127 were conducted according to Cañavate et al. (2006). The retention of VA in enriched
128 *Artemia* metanauplii in larval rearing tanks during the first four hours of starvation post-
129 enrichment did not change (Fernández, unpublished data). From 33 dph to the end of
130 the experiment (48 dph), larvae were progressively weaned onto dry feed (Gemma
131 Micro 150-300© Skretting, Spain).
132 The effect of VA in Senegalese sole skeletogenesis was evaluated by means of
133 four different dietary regimes containing graded levels of VA and using enriched
134 *Artemia* metanauplii as carrier; each regime was done in triplicate. As live preys
135 (rotifers and *Artemia* nauplii) accumulate VA in different patterns (Giménez et al.,
136 2007), we could not maintain the same levels of VA during the whole live prey-feeding
137 period. Thus, we decided to focus our study only during the *Artemia*-feeding phase.
138 The graded levels of VA in *Artemia* metanauplii were obtained by adding different
139 amounts of retinyl palmitate (1,600,000 IU g^{-1}, Sigma-Aldrich, Spain) to a commercial
140 enriching emulsion, Easy Selco™. Experimental emulsions were designed to contain

141 500 (D1), 1,000 (D2), 2,100 (D3) and 4,000 (D4) retinol equivalents g^{-1} (Table 1). For
142 comparative purposes, the emulsion containing 500 retinol equivalents g^{-1} (1,666 IU VA
143 g^{-1}) was considered as the control group (ES without retinyl palmitate). Both live preys
144 were enriched as previously described in Fernández et al. (2008).
145 Different parameters were measured in order to evaluate the effects of increasing
146 dietary VA levels on larval performance: retinoid content in enrichment emulsions, live
147 prey and larvae; larval growth (in length and weight) and survival rate; metamorphosis
148 (eye migration), bottom settlement and thyroid gland development (size and number of
149 follicles); maturation of the digestive system; and incidence of pigmentation disorders
150 and skeletal deformities. Larvae were sampled and sacrificed with an overdose of
151 anaesthetic (Tricaine methanesulfonate, MS-222, Sigma) at different ages from 2 to 48
152 dph, depending on the parameter considered.
153
154 *2.2 Biochemical analysis*
155 The retinoid content of the enrichment emulsions, enriched *Artemia* metanauplii, and
156 larvae was analyzed by HPLC, using a modified version of the method by Takeuchi et
157 al. (1998). After sampling, live prey and larvae were washed with distilled water to
158 remove salt and bacteria, and the samples were frozen at –80 ºC until posterior
159 analysis. Lipids were extracted with chloroform:methanol (C:M, 2:1) according to
160 Folch's method (Folch et al., 1957) and stored in C:M:BHT (2:1:0.01%) at 20 mg l^{-1} and
161 -20 °C until analysis. Lipid extracts were then evaporated and redissolved in
162 methanol:acetone (1:1, v/v) prior to HPLC analysis. The HPLC system (Thermo
163 Separation Products, San Jose, CA, USA) was equipped with a Lichrospher C-18
164 reversed-phase column (Merck, Darmstadt, Germany) and a UV–visible detector set at
165 a wavelength of 325 nm. The mobile phase was a mixture (85:15, v/v) of methanol
166 (98%) with 0.5% ammonium acetate and chloroform. The flow rate was 1.5 ml min^{-1},
167 and the elution time was 18 min. The concentration of each retinoid was calculated
168 from calibration curves constructed with the peak area ratios of their external standards

169 and an internal standard of retinol acetate added to the samples. All the reference
170 retinoids were purchased from Sigma-Aldrich (Spain).
171 The specificity of the method for the different retinoid compounds is guaranteed
172 by the retention times of the peaks in the standard injections and the lack of interfering
173 peaks in the blank runs. The four point linear regressions of the peak area and the
174 concentration ratios of the internal standard and each retinoid analysed had r^2 higher
175 than 0.9886, and were considered linear in the range of the tested samples. The
176 repeatability was assessed through the injection of five different standard solutions with
177 a mixture of the retinoids analysed for each of the four levels used in the calibration
178 curves. The coefficient of variation was in all cases below 5%. These standard
179 analyses also allowed checking the % recovery of the assayed retinoids, which was
180 found between 92 and 101%. No peak was considered below a signal/noise ratio of 10.
181
182
183 *2.3 Larval growth and survival rate*
184 At 2, 5, 10, 15, 20, 31, 41 and 48 dph, fifteen larvae from each tank were randomly
185 sampled, rinsed with distilled water, and used for body size and dry weight
186 determination. Larval standard length (SL) was measured with a digital camera
187 connected to a binocular microscope Nikon SMZ 800 and an image analysis system
188 (AnalySIS, Soft Imaging Systems, GmbH). Once larvae were measured in length, they
189 were dried at 60 ºC until their weight was constant. Samples were weighed with an
190 analytic microbalance (Sartorius BP211D). Survival rate was calculated as the
191 percentage of final surviving fish with respect to the initial number at the beginning of
192 the trial minus those individuals removed for sampling.
193
194 *2.4 Maturation of the digestive system*
195 The specific enzyme activity of two intestinal brush border enzymes (alkaline
196 phosphatase and aminopeptidase) and two pancreatic enzymes (trypsin and amylase)

was used to assess the degree of development and maturation of the digestive system of larvae fed graded levels of VA. Enzyme activity was measured at 15, 31, 41 and 48 dph (sampling size was 40, 30, 15 and 10 individuals per tank, respectively).

Sampled fish were washed with distilled water and stored at –80 ºC prior to enzyme activity analysis. All fish were dissected to separate pancreatic and intestinal segments as described by Cahu and Zambonino-Infante (1994). Samples were homogenized (Ultra-Turrax D25 basic, IKA© - Werke) in five volumes (v/w) of ice-cold Milli-Q water and centrifuged at 3,300 g (3 min) at 4 ºC, and the supernatant was removed for pancreatic enzyme quantification. Intestinal brush border membranes for determination of intestinal enzymes were purified according to Crane et al. (1979).

Trypsin (E.C. 3.4.21.4) activity was assayed at 25 ºC using BAPNA (N-α-benzoyl-DL-arginine *p*-nitroanilide) as substrate (Holm et al., 1988). Amylase (E.C. 3.2.1.1) activity was measured using soluble starch (0.3%) dissolved in Na_2HPO_4 buffer pH 7.4 as substrate (Métais and Bieth, 1968). Alkaline phosphatase (E.C. 3.1.3.1) was quantified at 37 ºC using 4-nitrophenyl phosphate (PNPP) as substrate (Bessey et al., 1946). Aminopeptidase N (E.C.3.4.11.2) was determined at 25 ºC according to Maroux et al. (1973) using sodium phosphate buffer 80 mM (pH = 7.0) and L-leucine *p*-nitroanilide as substrate (in 0.1 mM DMSO). Enzymatic activities were expressed as specific enzyme activity, in milliunits per milligram of protein (mU/mg protein), and soluble protein of crude enzyme extracts was quantified by means of the Bradford's method (Bradford, 1976) using bovine serum albumin as standard. All the assays were conducted in triplicate.

2.5 *Metamorphosis and bottom settlement*

Metamorphosis and settlement are two separate processes in flatfish development that might coincide in time depending on the species (Geffen et al., 2007). Thus, we used the term metamorphosis to define morphological and physiological development and the term settlement to define behavioural changes associated with the transition of

larvae from a planktonic to a benthonic way of life. Eye migration in Senegalese sole larvae is generally used as a measure of their metamorphosis progress. In this study, eye migration was evaluated at 10, 19, 20 and 30 dph (*n* = 200 larvae per dietary treatment) as in Fernández-Díaz et al. (2001). Data are presented as the relative amount of larvae at each stage of development at the same age. At the same sampling dates, digital photographs of the rearing tanks were taken in order to count the amount of swimming larvae in the water column and those at the bottom of the tank using image analysis software (AnalySIS).

The development of the thyroid gland (number and size of follicles) was evaluated in samples of 10, 15, 20, 30, 41 and 48 dph larvae (*n* = 10 larvae per rearing tank; *n* = 30 per dietary treatment). For histological purposes, larvae were processed according to standard histomorphological methods and stained with haematoxylin-eosin. Detection and semiquantification of thyroidal hormones, thyroxin (T_4) and triiodothyronine (T_3), was conducted according to Ortiz Delgado et al. (2006). At the end of the trial, three hundred and fifty specimens from each tank were examined to evaluate the effect of VA on juvenile pigmentation. Pigmentation in the ocular side was visually assessed by means of individual examination of all specimens, and pigmentary disorders were categorized according to the twelve categories described by Haga et al. (2002).

2.6 Skeletal deformities analysis, observations and measurements

To identify and quantify the skeletal deformities of larvae from the different dietary treatments, 50-60 larvae per tank were sampled at the end of the experiment and fixed in formaldehyde solution (10%) until double stained. Animals were stained for bone and cartilage in whole mount preparations using a modification of the method described by Klymkowsky and Hanken (1991).

After staining, fish were placed on their blind (left) side to observe meristic characters and skeletal abnormalities in the cranium, vertebral column, and caudal fin

253 complex. Skeletal structures were identified and named according to Gavaia et al.
254 (2002) and Wagemans and Vandewale (2001). The study focused on the mean
255 number of vertebrae and the frequency of individuals with an abnormal number of
256 vertebrae. Special attention was given to the deformities occurring in the cranial region,
257 vertebral column, and caudal fin complex (hypurals, parahypural, epural, modified
258 haemal spines and modified neural spine).
259
260 *2.7 Statistical analysis*
261 Results are given as mean and standard deviation. Data expressed as percentage
262 (survival, incidence of skeletal deformities, eye migration success, pigmentary
263 disorders, and larval bottom settlement) were previously arcsin($x^{1/2}$)-transformed. All
264 data were checked for normality (Kolmogorov–Smirnov test) and homoscedasticity of
265 variance (Bartlett's test) and then compared by means of One Way ANOVA (Zar,
266 1974). When significant differences were detected, the Tukey multiple-comparison test
267 was used to detect differences among experimental groups. Correlation between
268 different variables was evaluated with the Pearson Product Moment Correlation test. In
269 all statistical analyses, the level of significant difference was set at $P < 0.05$. All the
270 statistical analyses were conducted using SigmaStat 3.0 (Systat Software Inc.,
271 Richmond, USA).
272
273
274 **3. Results**
275
276 *3.1 Retinoid content in experimental emulsions and live prey*
277 Table 1 presents the total lipid and total VA content (retinol and retinyl palmitate) in
278 experimental emulsions used for enriching *Artemia* metanauplii with graded levels of
279 retinyl palmitate. No statistically significant differences were detected in the total lipid
280 content of experimental emulsions containing different levels of VA (ANOVA, $P > 0.05$).

281 Total VA content in the emulsions increased with increasing levels of retinyl palmitate
282 incorporated (ANOVA, $P < 0.05$).
283 The retinoid content and total VA of enriched *Artemia* metanauplii is shown in
284 Figure 2. The HPLC analysis revealed that the main retinoid found in enriched *Artemia*
285 was retinyl palmitate (VA ester), representing between 67 to 76% of the total VA
286 content. The retinyl palmitate concentration increased in enriched *Artemia* with
287 increasing levels of this compound in the enriching emulsion (ANOVA, $P < 0.05$). The
288 level of retinyl palmitate in live prey increased up to 5.1 times when we compared
289 *Artemia* enriched with D1 and D4 (7.7 and 39.4 ng mg^{-1} DW, respectively). The content
290 of retinol (VA alcohol) in enriched live prey followed a similar pattern. While the retinol
291 content of *Artemia* enriched with D1 and D2 was not significantly different, its level
292 increased from 3.4 to 15.3 ng mg^{-1} DW (4.5-fold increase) (ANOVA, $P < 0.05$) in D1-
293 and D4-enriched *Artemia*, respectively. In contrast, the retinoic acid content in *Artemia*
294 enriched with D4 was 16.8 times higher than in *Artemia* enriched with D1 and D2, in
295 which it increased from 0.37 to 6.2 ng mg^{-1} DW, respectively. *Artemia* enriched with D3
296 showed intermediate levels of retinoic acid accumulation (1.0 ng mg^{-1} DW; 2.8-fold
297 increase in relation to the control group) (ANOVA, $P < 0.05$). Retinal (aldehyde form of
298 VA) was not detected in *Artemia* enriched with graded levels of VA.
299
300 *3.2 Retinoid content in larvae*
301 Figure 3 shows the retinoid (retinol and retinyl palmitate) content in Senegalese sole
302 larvae fed different VA regimes between 2 and 48 dph. During the study, retinyl
303 palmitate increased as a consequence of the level of this retinoid in *Artemia*, whereas
304 retinol showed the opposite trend and decreased to 55% as compared to its content in
305 2-dph larvae.
306 At the end of the study, the accumulation of retinyl palmitate and retinol in early
307 juveniles was linked to the level of total VA administered during the *Artemia* feeding
308 phase ($r^2 = 0.97$ and 0.99, respectively; $P < 0.001$, Pearson Product Moment

309 Correlation test). However, only the values of retinyl palmitate and retinol body content
310 in fish fed D4-enriched *Artemia* were significantly higher than the mean value from the
311 rest of dietary groups (27.75 ± 2.68 *vs*. 22.61 ± 0.25 ng retinyl palmitate mg^{-1} DW and
312 0.88 ± 0.07 *vs*. 0.70 ± 0.03 ng retinol mg^{-1} DW; $P < 0.05$, ANOVA).
313
314 *3.3 Larval growth and survival*
315 At 10 dph, larvae fed D1-, D2-, and D3-enriched *Artemia* were significantly larger than
316 larvae fed the diet containing the highest content of total VA (D4) (Fig. 4a; ANOVA, $P <$
317 0.05). However, no differences in larval size were detected at older ages (15, 20 and
318 31 dph) until 41 and 48 dph, coinciding with the weaning phase. At 41 and 48 dph, fish
319 fed *Artemia* enriched with the control emulsion (D1) were larger than those from the
320 rest of the dietary groups (Table 2; ANOVA, $P < 0.05$). Dry weight was not significantly
321 affected by any of the dietary treatments at any sampling time of the experiment (Fig.
322 4b; ANOVA, $P > 0.05$). Different levels of total VA in enriched *Artemia* did not affect
323 Senegalese sole larval survival at the end of the study (Table 2; ANOVA, $P > 0.05$).
324
325 *3.4 Maturation of the digestive system*
326 Figure 5 shows changes in the enzyme specific activity of selected pancreatic and
327 intestinal enzymes from fish fed the control diet (D1). From 15 to 48 dph, the specific
328 activity of amylase gradually decreased from 0.619 to 0.014 U mg $protein^{-1}$ (ANOVA, P
329 < 0.05). A 2.8-fold decrease in trypsin specific activity was also observed between 15
330 (0.398 mU mg $protein^{-1}$) and 30 dph (0.145 mU mg $protein^{-1}$), remaining fairly constant
331 until the end of the study. However, alkaline phosphatase specific activity was constant
332 from 15 to 41 dph (4.02 U mg $protein^{-1}$) but showed a 2.2-fold increase at 48 dph (8.86
333 U mg $protein^{-1}$). In contrast, aminopeptidase-N specific activity remained constant
334 throughout the studied period (mean value of 0.089 mU mg $protein^{-1}$). Different levels
335 of VA did not affect the specific activity of pancreatic or intestinal enzymes at any
336 sampling point considered (ANOVA, $P > 0.05$). At 41 and 48 dph, trypsin, alkaline

337 phosphatase and aminopeptidase-N specific activity tended to be lower in fish fed D3
338 and D4 in comparison to fish fed D1 and D2, although this reduction in enzyme activity
339 was not statistically significant (data not shown).
340
341 3.5 *Metamorphosis and bottom settlement*
342 Results of thyroid gland development are presented in Table 3. In 10- and 15-dph
343 metamorphic larvae, dietary VA levels affected the number of thyroid follicles, although
344 not significantly (ANOVA, $P > 0.05$). The intensity of the immunoreactive staining of T_3
345 and T_4 hormones showed no differences between the above-mentioned larval ages
346 (Table 4). At older stages of development (20, 30, 41 and 48 dph post-metamorphic
347 larvae), the increase in dietary VA reduced the number of follicles while increasing their
348 average size (ANOVA, $P < 0.05$). These changes in the development of the thyroid
349 glands concurred with an increase in the immunoreactive staining of T_3 and T_4
350 hormones (Fig. 6).
351 Bottom settlement was a fast process in Senegalese sole larvae that coincided
352 with metamorphosis (eye migration). At 20 dph all fish had settled to the bottom, and
353 most of them had completed eye migration. The level of VA in enriched *Artemia* did
354 only significantly affect the process of settlement in metamorphosing larvae at 9 dph,
355 when fish fed D3 and D4 showed higher rates of benthic larvae (8.6 ± 2.9%) in
356 contrast to those from D1 and D2 groups (6.1 ± 1.8%) (ANOVA, $P < 0.05$). No
357 significant differences in the rate of benthic larvae were detected at older ages among
358 different experimental groups (12 dph: 66.0 ± 8.8%; 14 dph: 89.5 ± 4.0%; 19 dph: 96.4
359 ± 1.4%; 20 dph: 100%; data shown as the mean value of all experimental groups). Eye
360 migration results are shown in Figure 7. The onset of eye migration started earlier in
361 fish fed D2, D3 and D4 than in the control group. At 10 dph, the D2, D3, and D4 groups
362 showed a higher frequency of specimens in stage 1 than the control group (23.8 vs.
363 2.2%, respectively; ANOVA, $P < 0.05$). However, these differences were not evident at
364 older ages (19, 20 and 30 dph). Also, no differences in the frequency of fish at further

365 stages of eye migration (stages 2-4) were detected among different dietary
366 experimental groups (ANOVA, $P > 0.05$). At 30 dph, eye migration process was
367 completed (stage 4) and any case of abnormal eye migration was recorded in any of
368 the experimental groups.
369 At the end of the study, the rate of fish exhibiting pigmentation problems was
370 the same for all the dietary groups (ANOVA, $P > 0.05$), with an average incidence of
371 pseudoalbinism of 2.3 ± 1.0%. Ambicolouration was not observed in any of the
372 sampled fish fed different levels of VA.
373
374 *3.6 Skeletal deformities: typology and frequencies*
375 Dietary levels of VA directly affected skeletogenesis and the incidence of skeletal
376 deformities in Senegalese sole (Figure 8a). The frequency of deformed specimens
377 increased with the dietary dose of VA, as well as the incidence of fish with more than
378 one deformity in their skeleton (ANOVA, $P < 0.05$). In particular, the incidence of
379 deformities ranged from fish with only one small skeletal abnormality to fish displaying
380 multiple deformities with different degrees of severity (Fig. 8b; Fig. 11).
381 Cranial deformities (26.7%) were only observed in fish fed D4 (Figure 8c). The
382 structures mostly affected were those related with the opercular complex, especially
383 the preopercular, interopercular, ceratohyal, and ceratobranchials 1-5. The incidence of
384 cranial deformities in the D4 group was significantly correlated to the presence of
385 deformed prehaemal vertebrae numbers 1-3 ($r^2 = 0.998$, $P = 0.002$). No skeletal
386 deformities were observed in the jaw apparatus and neurocranium in any of the dietary
387 treatments.
388 The vertebral column was composed of 45 vertebrae, divided in 8 prehaemal
389 and 37 haemal vertebrae (including the urostyle). No significant differences were
390 detected in the mean number of specimens with 44, 45 and 46 vertebrae (ANOVA, $P >$
391 0.05) among the different VA treatments. However, the incidence of a supranumerary

392 vertebra was higher in fish fed D2, D3 and D4 than in those fed D1 (36.0, 38.0 and
393 44.4 vs. 28.0%, respectively; $P = 0.02$).
394 Figure 9 shows the incidence of deformities along the vertebral column axis. In
395 all experimental groups, most of deformities affecting the axial skeleton where
396 observed between the vertebra number 38 and the urostyle; whereas increasing
397 dietary levels of VA increased the incidence of deformities in the prehaemal vertebrae.
398 Skeletal abnormalities in the vertebral column (prehaemal and haemal regions)
399 increased with increasing levels of dietary VA in enriched Artemia ($r^2 = 0.981$, $P =$
400 0.018; Fig. 10a). Torsion of the first three prehaemal (cephalic) vertebrae (14%) was
401 recorded only in fish fed the highest dose of VA (D4). This type of deformity consisted
402 of a change in the morphology of the vertebral disk resulting in a realignment of the
403 axial skeleton and a slight torsion of the basioccypital articulatory process (Fig. 11b).
404 The frequencies of deformities in prehaemal and haemal vertebral centrums (fusion
405 and compression) were significantly affected by the level of VA in the diet (Fig. 10b, c),
406 although prehaemal centrums were less affected than haemal ones (16.7 vs. 63.3% in
407 fish fed D1). No significant differences were detected in the incidence of deformities
408 affecting the prehaemal centrums among fish fed D1, D2 and D3 diets (18.4%;
409 ANOVA, $P > 0.05$), whereas the incidence of deformities in haemal vertebrae
410 significantly increased with the level of dietary VA (ANOVA, $P < 0.05$). However, the
411 frequency of fish with abnormal prehaemal and haemal centrums significantly
412 increased 3.2 (59.3%) and 1.5 times (97.3%), respectively, in fish fed D4 (ANOVA, $P <$
413 0.05), indicating that prehaemal centrums were more sensitive than haemal centrums
414 to dietary levels of VA.
415 Vitamin A also significantly affected the incidence of deformed neural and
416 haemal spines (Fig. 10d and e; ANOVA $P < 0.05$). Figures 11c and 11d show different
417 typologies of deformities affecting vertebral spines. The frequency of both abnormal
418 neural spines and haemal spines was similar between fish fed D1 and D2 (78.4 and
419 71.4%, respectively), whereas it progressively increased in fish fed D3 (86.7 and 80.7,

420 respectively) and D4 diets (98.7 and 92.7%, respectively), showing significant
421 differences (ANOVA, $P < 0.05$). The incidence of deformed parapophyses increased
422 from 19.3% in fish fed D1 up to 50.7% in fish fed D4 (2.6-fold increase; Fig. 10f),
423 whereas those specimens fed D2 and D3 showed intermediate values of abnormal
424 parapophyses (35.0%).
425 The dietary VA level affected all the skeletal structures composing the caudal
426 fin complex, although the incidence of deformities varied depending on the structure
427 considered and the dose of VA (Fig. 12a). The most common deformity affecting the
428 parahypural and the hypurals (1-5) was the fusion of these structures with those
429 adjacent, which produced changes in their regular shape (Fig. 11e-h). The occurrence
430 of abnormal hypurals increased with high levels of dietary VA. The incidence of fish
431 with abnormal hypurals almost doubled, from 36.7% in fish fed D1 up to 66.0% in those
432 fed D4. Fish fed D2 and D3 showed intermediate values of abnormal hypurals (48.7%),
433 with no significant differences between them (ANOVA $P < 0.05$; Fig. 12c). The
434 incidence of abnormal parahypural was similar among fish fed *Artemia* enriched with
435 D1, D2, and D3 (13.3% average value for the three treatments), which was significantly
436 lower than in fish in the D4 group (41.3%; Fig. 12b; ANOVA, $P < 0.05$). The incidence
437 of deformed (twisted) epural in fish fed D2 and D3 (31.0%) showed a 1.9-fold increase
438 in relation to fish from the control group (16.0%), whereas this rise was 3.7 times higher
439 in fish fed D4 (58.7%; Fig. 12d). No significant differences were detected in the
440 incidence of deformities affecting the modified neural spine between D1, D2, and D3
441 groups (40.4%; ANOVA, $P > 0.05$), whereas in the D4 group the number of fish with
442 abnormal modified neural spine significantly increased 1.7 times (68.0%; Fig. 12e;
443 ANOVA, $P < 0.05$). The frequency of abnormal modified haemal spines (1-2) tended to
444 increase with increasing levels of dietary VA (Fig. 12f), being 2.3 times higher in fish
445 fed D4 than in those fed D1. A significant increase of 1.4- and 1.9-fold was recorded in
446 fish fed D2 and D3, respectively (ANOVA $P < 0.05$).
447

448

449 **4. Discussion**

450

451 The effects of different levels of VA in Senegalese sole larval development were
452 studied by means of a dose-response experiment using enriched *Artemia* metanauplii
453 as carrier. Although the use of microdiets in co-feeding rearing protocols for the early
454 weaning of Senegalese sole has been greatly improved (Fernández-Díaz et al., 2006;
455 Engrola et al., 2009), we decided to bioencapsulate VA in live prey because
456 Senegalese sole larvae cannot be fed exclusively with microdiets. As previously shown
457 (Giménez et al., 2007), total VA in *Artemia* metanauplii accumulated proportionally to
458 the content of retinyl palmitate in the enriching emulsions. Although retinoic acid was
459 absent in the original emulsion, its presence in the metanauplii enriched with the
460 highest levels of VA (D3 and D4) indicated that live prey were able to metabolize
461 different retinoids and oxidize retinol into retinoic acid. Since retinoic acid is a much
462 more active VA metabolite than the other retinoids (Ross et al., 2000), interpreting the
463 results from the dose-response experiment must take into consideration its presence in
464 D3- and D4-enriched *Artemia* metanauplii. The retinoid content in live prey directly
465 affected the accumulation of VA in the larvae and, especially, in early juveniles, as
466 retinyl palmitate and retinol body contents clearly showed. Of the two forms of VA,
467 retinyl palmitate was the dominant form accumulated in Senegalese sole tissues.
468 Under our experimental conditions, retinyl palmitate accumulated during larval
469 ontogeny, whereas retinol showed the opposite trend, decreasing from hatching until
470 41 dph and then remaining constant until the end of the study. Retinyl esters, the main
471 form of retinoids in live prey, are hydrolyzed into retinol in the lumen of the larval
472 digestive tract, absorbed by the enterocytes, re-esterified, and transported to the liver
473 through the lymphatic system by chylomicrons. Once in the liver, the main site for VA
474 body storage, retinyl esters are hydrolyzed and re-esterified again in retinyl palmitate,
475 which is finally stored in hepatocytes (Hamre et al., 2005). Thus, the accumulation of

476 retinyl palmitate in Senegalese sole larvae would reflect the dose-dependent
477 accumulation of this form of VA due to the experimental feeding treatments and the
478 larval age. In contrast, ontogenetic changes in both VA metabolism and larval
479 requirements might explain the decrease in retinol content during the experimental
480 period, since this form of VA and retinal constitute the total VA content in eggs and
481 newly hatched larvae, with their content decreasing with larval development and
482 metamorphosis (Moren et al., 2004a).
483 In fish species, VA requirements for normal development and optimal growth present
484 inter-specific differences. Thus, in Japanese flounder (Dedi et al. 1997; Haga et al.
485 2003), Atlantic salmon (Ørnsrud et al. 2002), European sea bass (Villeneuve et al.,
486 2005, 2006), red sea bream (Hernández et al., 2006), and gilthead sea bream
487 (Fernández et al., 2008), high dietary doses of VA during larval development lead to
488 poor growth performance and survival. Surprisingly, we found that Senegalese sole
489 larval survival and growth, in terms of body weight, were not affected by the dietary VA
490 content, and differences in total length were only observed after the weaning.
491 Therefore, high levels of VA were not toxic (hypervitaminosis A) in terms of final growth
492 in weight and survival of the fish, and the smaller size of the fish might be a
493 consequence of a higher incidence of deformities in the caudal region of their vertebral
494 column (Haga et al., 2002). According to the National Research Council, the
495 requirements of VA for juveniles of different fish species, such as rainbow trout,
496 salmon, channel catfish and sea bream, ranged between 1,000 and 3,500 IU kg^{-1}
497 (NRC, 1993). In contrast, when considering different flatfish species, the safe level of
498 VA in *Artemia* nauplii for preventing the development of skeletal abnormalities in
499 Japanese flounder was less than 45,200 IU VA kg^{-1} (Dedi et al., 1995). In summer
500 flounder and Atlantic halibut juveniles fed microdiets containing different levels of VA, a
501 diet containing less than 52,873 and 8,333 IU VA kg^{-1} respectively has been described
502 as the best for assuring a proper juvenile development (Lewis-McCrea and Lall, 2007;
503 Moren et al., 2004b, respectively). Under present experimental conditions, Senegalese

504 sole larvae fed *Artemia* metanauplii enriched with a commercial emulsion containing
505 4,333 IU kg^{-1} showed a high incidence of skeletal abnormalities, which seems to
506 indicate that this species is quite sensitive to low dietary levels of this nutrient.
507 However, published results regarding the VA requierements in different fish species
508 might be taken cautiously, since there might be differences depending on the stage of
509 development of experimental fish (larva vs. juvenile), the type of retinoid compound
510 included into the diet (retinyl esters, retinoic acid or carotenoids), the experimental
511 design, the rearing conditions or the analytical method for VA quantification.
512 Thyroid hormones, VA, and fatty acids are all factors that have been shown to
513 affect metamorphosis in flatfish by disrupting the normal pigmentation and eye
514 migration patterns (see reviews by Hamre et al., 2005, 2007). Several authors have
515 described hyperpigmentation (Martínez et al., 2002) or improved pigmentation (Estévez
516 and Kanazawa, 1995; Takeuchi et al., 1995; Dedi et al., 1997; Haga et al. 2002) of
517 flatfish larvae fed live prey enriched with VA, although in some studies high VA levels
518 increased the frequency of skeletal deformities. Under the present conditions, dietary
519 VA did not affect pigmentation patterns in Senegalese sole. This might indicate a
520 species-specific sensitivity to a dietary excess of VA in the differentiation of pigmentary
521 cells that may either differentiate into adult melanonophores or disappear by apoptotic
522 processes (see review in Bolker and Hill, 2000).
523 In addition, dietary levels of VA did not alter the process of settlement in
524 metamorphosing Senegalese sole larvae, although they affected eye migration in early
525 metamorphosis (10 dph). Thus, 10-dph larvae fed high levels of VA (D2, D3, and D4
526 groups) showed a precocious formation of the ocular channel and the initiation of eye
527 migration. These differences were not observed in the latter stages. Senegalese sole
528 presents a narrow size threshold for the onset of metamorphosis, resulting in a
529 synchronised settling behaviour and a uniform post-settlement size distribution
530 (Fernández-Diaz et al., 2001). Thus, the high frequency of larvae in early stages of
531 metamorphosis at 10 dph might be associated with their larger size, since

532 metamorphosis in this species depends on larval size (see review in Geffen et al.,
533 2007) and the levels of thyroid hormones (Ortiz Delgado et al., 2006; Klaren et al.,
534 2008).

535 Pancreatic and intestinal enzyme activity provides a reliable marker of larval fish
536 development (Zambonino Infante et al., 2008). In the present study, an excess of
537 dietary VA did not affect the activity levels of these digestive enzymes in Senegalese
538 sole larvae, which followed the general trend previously described for this species
539 (Ribeiro et al., 1999). In contrast, gilthead sea bream (Fernández et al., 2008) and
540 European sea bass (Villeneuve et al., 2005, 2006) larvae fed high doses of VA showed
541 a delay in the maturation of their digestive function. Thus, we can hypothesize that the
542 levels of VA tested in the present experiment are sublethal, since they did not affect the
543 overall development of Senegalese sole larvae, neither in terms of larval survival, body
544 weight, nor maturation of the digestive function. On the other hand, dietary VA levels
545 affected dramatically the normal process of bone formation and skeletogenesis in
546 Senegalese sole larvae.

547 Different studies have shown a high incidence of skeletal deformities in
548 hatchery-reared early juveniles of Senegalese sole, ranging from 44% (Gavaia et al.,
549 2002) to 80% (Engrola et al., 2009). In our study, fish fed the control diet also showed a
550 high frequency of individuals with deformed skeletal structures. Furthermore, an
551 increase of dietary VA resulted in a significant increase in deformities. The incidence of
552 skeletal deformities reported in Senegalese sole reared under standard feeding
553 protocols is higher than that observed in other commonly produced species in the
554 Mediterranean area, like gilthead sea bream (Boglione et al., 2001; Fernández et al.,
555 2008) or European sea bass (Villeneuve et al., 2005; Mazurais et al., 2008). Two
556 different hypotheses might explain such a high incidence of skeletal deformities in
557 Senegalese sole. The first considers that this flatfish species is more prone to develop
558 skeletal disorders than other fish species under any rearing conditions. The second
559 hypothesis postulates that since the skeletal deformities observed in Senegalese sole

were not lethal, higher final numbers of Senegalese sole specimens with deformities would be observed at the juvenile stage. Consequently, the observed incidence of deformities in Senegalese sole early juveniles was higher than in those species where deformities were lethal at early stages (Divanach et al., 1997; Koumoundouros et al., 1997; Boglione et al., 2001). Since both hypotheses are not mutually exclusive, determining which of the two models better explains the observations requires further developmental studies that would identify the most sensitive periods of morphogenesis and skeletogenesis to the development of deformities, as well as the timing of appearance of the deformities and their impact on larval survival.

The skeletal structures most affected by high dietary levels of VA in Senegalese sole were those from the vertebral column and caudal fin complex. Previously published studies found that several structures from the splanchnocranium, such as the premaxilla, maxilla and dentary bones, were the structures most severely affected by dietary VA (Haga et al., 2002, 2003; Villeneuve et al., 2005, 2006; Fernández et al., 2008). However, these skeletal structures did not show any changes in fish fed experimental diets in the present study. Since the diets with VA in excess were not offered to the larvae until 7 dph, when most of the pharyngeal skeleton was already ossified, the absence of changes in those skeletal structures is probably related to the timing of VA administration. In the present study, the opercular complex, in particular, the preopercular, interopercular, ceratohyal, and ceratobranchials 1-5, were mostly affected by the diet with the highest level of VA and retinoic acid (D4). The strong statistical correlation (Pearson Product Moment Correlation test) found between the deformed opercular structures and the cephalic vertebrae, suggests that the altered shapes of the opercular bones are a consequence of the torsion of the first three prehaemal (cephalic) vertebrae coupled with the restructuring processes of the cranial bones. These processes take place during eye migration and the completion of the typical asymmetrical body shape of this species; thus, the observed deformities seem to be more related to a disruption (acceleration) of the normal larval metamorphosis

588 pattern, rather than dietary VA acting directly on the above-mentioned opercular
589 elements.
590 Vitamin A impaired the development and number of vertebrae in Senegalese
591 sole. Similarly to Japanese flounder (Haga et al., 2002) and gilthead sea bream
592 (Fernández et al., 2008), high levels of dietary VA in Senegalese sole were responsible
593 for a higher incidence of a supranumerary vertebra in the haemal region of the
594 vertebral column of the fish. Contrastingly, in European sea bass an excess of VA
595 resulted in the loss of one vertebra (Villeneuve et al., 2006). In Senegalese sole, since
596 morphogenesis of the vertebral centrums follows a caudal direction (Gavaia et al.,
597 2002), vertebrae from the haemal region are the last ones to differentiate and ossify by
598 intramembranous ossification. The notochord is responsible for the proper
599 morphogenesis of the vertebral centrums, and consequently this tissue plays an
600 important role in inducing vertebral formation and maintaining vertebral morphogenesis
601 (Witten et al., 2005). Thus, dietary VA levels might have disrupted the segmentation of
602 the notochord and the normal process of morphogenesis in the vertebral centrums,
603 leading to a change in the number of vertebrae, as Haga et al. (2009) recently
604 demonstrated using transgenic zebrafish exposed to retinoic acid.
605 The impact of dietary VA on the incidence of skeletal deformities in different
606 regions of the vertebral column was also affected by the timing of the intramembranous
607 ossification. The first three prehaemal (cephalic) vertebrae, which are the first elements
608 of the vertebral column to ossify (Gavaia et al., 2002), were the least affected in
609 comparison to the rest of the prehaemal and haemal regions. Only deformed cephalic
610 vertebrae were detected in fish fed D4-enriched *Artemia* containing high levels of
611 retinyl palmitate and retinoic acid, whereas deformities affecting the rest of the
612 prehaemal and all the haemal vertebrae were detected in all experimental groups,
613 although at different prevalence rates. Therefore, the dose of VA and the timing of
614 morphogenesis directly affect the incidence of skeletal disorders (Villeneuve et al.,
615 2006; Mazurais et al., 2008). Skeletal deformities affecting prehaemal and haemal

616 vertebrae in Senegalese sole early juveniles included: compressed, deformed and
617 fussed centrums; alterations of the intervertebral space; and deformed (twisted)
618 parapophyses, neural and haemal spines, which were more frequent in the haemal
619 vertebrae of fish fed high doses of dietary VA. According to Gavaia et al. (2002), who
620 described the osteological development of the caudal complex and vertebral column in
621 Senegalese sole for the first time, the development of both vertebral column and
622 caudal fin complex begins at 12–13 dph (16-18 ºC). However, in the present study this
623 development might have occurred earlier due to the slightly higher rearing
624 temperatures. In this regard, the high incidence of deformities in the prehaemal and
625 haemal regions of the vertebral column seemed to be related to an abnormally early
626 differentiation pattern. Thus, a prolonged exposure to an excess of VA might have
627 altered the normal process of morphogenesis in those skeletal elements formed either
628 by chondral (neural and haemal spines) or by intramembranous (vertebral centrums)
629 ossification. This would enhance the appearance of skeletal disorders, as previously
630 described in Japanese flounder (Haga et al., 2002), Atlantic salmon (Ørnsrud et al.,
631 2002), European sea bass (Villeneuve et al., 2005, 2006), red sea bream (Hernández
632 et al., 2006), summer flounder (Martínez et al., 2007), and gilthead sea bream
633 (Fernández et al., 2008). Compressed vertebrae and reductions in the intervertebral
634 spaces might be associated with the presence of supranumerary vertebrae, as
635 reported for gilthead sea bream (Fernández et al., 2008). However, in Senegalese sole
636 the incidence of supranumerary vertebrae was not proportional to that of vertebral
637 compressions and fusions. These findings suggest that these skeletal deformities might
638 also be related to alterations in the areas of vertebral centrum growth and the failure of
639 notochord cells to maintain proper vertebral development and growth, as described in
640 Atlantic salmon (Witten et al., 2005).
641 The caudal fin complex was the most altered region of the Senegalese sole
642 skeleton, although the incidence of deformities varied depending on the structure
643 considered and the dose of VA. Although the deformities affected all the skeletal

644 elements composing the caudal fin, the most affected structures, in order of
645 importance, were the modified neural and haemal spines, epural, hypurals, and
646 parahypural. These results are in agreement with those observed in Japanese flounder
647 (Dedi et al., 1998) but differ from those reported in gilthead sea bream fed an excess of
648 VA, where the most affected caudal bones were the epurals, hypurals, parahypural,
649 neural arch, and uroneurals (Fernández et al., 2008). The differences between both
650 flatfish species and gilthead sea bream might be due to species-specific patterns in the
651 morphogenesis of the caudal complex linked to metamorphosis and the acquisition of
652 asymmetry and benthic life. Considering previous descriptions of the osteological
653 development of the caudal fin complex (Gavaia et al., 1999; 2006) and the results
654 obtained in Japanese flounder and gilthead sea bream larvae fed graded levels of VA
655 (Dedi et al., 1998; Fernández et al., 2008, respectively), the differences in sensitivity to
656 dietary VA amongst caudal fin skeletal elements might be due to differences in their
657 ontogenetic development and the duration of VA exposure. The high incidence of
658 fusion between hypurals and parahypural has also been observed in Japanese
659 flounder early juveniles (Dedi et al., 1998). Thus, VA might have stimulated the
660 differentiation and proliferation of chondrocytes (hypertrophic differentiation) in the
661 above-mentioned structures, leading to their fusion due to their close proximity and
662 their almost simultaneous temporal development (Gavaia et al., 2002).
663 Up to this point, we have only considered the effect of VA on Senegalese sole
664 skeletogenesis by its direct action through retinoic acid in the skeletal tissue. However,
665 present results indicate that VA also affected the levels of thyroid hormones T_3 and T_4
666 in Senegalese sole larvae. Thyroid hormones are essential regulators of skeletal
667 development and bone maintenance (Wexler and Sharretts, 2007). During
668 development, thyroid hormones, especially T_3, are essential for the recruitment and
669 maturation of bone cells. In mammals, alterations in the thyroid status result in
670 acceleration of bone formation (by either chondral or intramembranous ossification),
671 growth abnormalities, bone loss, and increased fracture risk (Harvey et al., 2002). In

672 particular, excessive amounts of thyroid hormone induce increased activity of
673 osteoblasts and osteoclasts leading to high bone turnover and loss of bone mineral
674 density, as the activity of osteoclasts predominates over the activity of osteoblasts
675 (Mikosch, 2005). The action of thyroid hormones on the development and health of the
676 skeletal tissue is mediated by nuclear receptor proteins (TR), which are expressed in
677 chondrocytes and osteoblasts. These proteins are members of the superfamily of
678 hormone and orphan nuclear receptors and function as hormone-inducible transcription
679 factors (Harvey et al., 2002). The TR proteins together with retinoid X receptors form
680 heterodimers (RXR) that bind to specific T_3-response element sequences within target
681 gene promoters and modulate their transcriptional regulation (Duncan Basset et al.,
682 2007). Thus, there is a convergence of VA- and thyroid hormone receptor-mediated
683 pathways on bone formation and remodelling. Although Senegalese sole has an
684 acellular bone, the mechanisms of bone tissue formation and growth are quite
685 conserved among vertebrates and also their signalling pathways (Witten and
686 Huysseune, 2007), which implies that modifications in the thyroid hormone status might
687 have a direct effect on skeletal morphogenesis. Disruption of these pathways by either
688 dietary VA imbalances or changes in the levels of T_3 might affect the process of normal
689 skeletogenesis, leading to skeletal deformities.
690
691
692 **5. Conclusions**
693
694 Under the present experimental conditions and independently of the feeding treatment,
695 Senegalese sole exhibited high levels of skeletal abnormalities, particularly in the
696 vertebrae and caudal fin complex. Therefore, even the control group (fish fed *Artemia*
697 metanauplii enriched with a commercial emulsion) was exposed to a dietary dose of VA
698 that might have altered the harmonious development of the axial and caudal skeleton.
699 Thus, we need to conduct further research using emulsions with even lower levels of

VA (retinyl palmitate) to discriminate between the effects of this nutrient and other factors inducing skeletal disorders in Senegalese sole. In this regard, we need to evaluate the effect of other nutrients, such as essential fatty acids, minerals and vitamins (particularly liposoluble vitamins D, E, and K) (Lewis et al., 2007; Mazurais et al., 2008), genetic factors (Kacem et al., 2004), and/or unsuitable husbandry and rearing practices and rearing temperatures (Lewis et al., 2004; Blanksma et al., 2009; Sfakianakis et al., 2006), that might also have been affecting the skeletal development of Senegalese sole larvae. The inherent complexity of skeletogenesis is such that a holistic approach to discriminate and evaluate the relative importance of each of the above-mentioned factors is not possible, and consequently this question needs to be addressed in singular experiments.

Our studies on the effects of different dietary VA levels on Senegalese sole performance revealed that an excess of VA affected neither larval performance in terms of survival and growth nor the maturation of the digestive system. However, this morphogenetic nutrient had a remarkable impact in the skeleton morphogenesis. An excess of VA accelerated the intramembranous ossification of vertebral centrums, leading to a supranumerary haemal vertebra and a high incidence of fused and compressed vertebrae. In addition, VA also affected those structures from the vertebrae and caudal fin formed by chondral ossification, leading to defects in their shape and fusions with adjacent skeletal elements. However, we should not dismiss the impact of other systemic factors such as thyroidal hormones in skeletogenesis since in our studies an excess of dietary VA affected the levels of thyroid hormones (T_3 and T_4), which might have affected metamorphosis, bone formation and remodelling, leading to skeletal deformities.

Further studies are needed to identify the potential crosstalk between VA and thyroid hormones and their effects on the expression of different genes involved in Senegalese sole early morphogenesis and skeletogenesis.

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934

935 **Figure captions**

936 Figure 1. Feeding protocol of Senegalese sole. *Artemia* metanauplii were enriched with
937 experimental emulsions containing 500 (D1), 1,000 (D2), 2,100 (D3) and 4,000 (D4)
938 retinol equivalents g^{-1}.

939

940 Figure 2. Retinoid (retinoic acid, retinol, and retinyl palmitate) and total vitamin A
941 content (ng retinoid compound mg^{-1} DW) in *Artemia* metanauplii enriched with graded
942 levels of VA [500 (D1), 1,000 (D2), 2,100 (D3) and 4,000 (D4) retinol equivalents g^{-1}].
943 For comparative purposes, the mean value of the total VA content in enriched live prey
944 is included for each treatment. Different letters denote the existence of statistically
945 significant differences among the content of different compounds depending on the
946 treatment (ANOVA, $P < 0.05$).

947

948 Figure 3. Changes in body content of retinol and retinyl palmitate (ng retinyl palmitate
949 mg DW^{-1}) of Senegalese sole larvae fed graded levels of vitamin A. Different indexed
950 letters show significant differences between treatments (ANOVA, $P < 0.05$).

951

952 Figure 4. Growth in standard length (a) and dry weight (b) of Senegalese sole larvae
953 fed Artemia enriched with graded levels of VA. At 10 dph, the asterisk denotes the
954 existence of significant differences in standard length between groups (see text for
955 details). The dotted line represents the onset of the weaning period. Different letters
956 indicate statistically significant differences among dietary treatments (ANOVA, $P <$
957 0.05)

958

959

960 Figure 5. Changes in specific enzyme activity of trypsin (a), amylase (b), alkaline
961 phosphatase (c), and aminopeptidase-N (d) in Senegalese sole fed the control diet.

962 Different letters denote the existence of statistically significant differences among
963 different sampling points (ages).
964
965 Figure 6. Immunolocalization of T_3 and T_4 in Senegalese sole larvae fed different levels
966 of vitamin A (haematoxylin and eosin/peroxidase staining). Thyroid follicles in a 15-dph
967 larva from D1 (a) and D3 (b). Note the presence of a small follicle at the base of the
968 aortic bulb (arrowhead); (c) and (d), thyroid follicles of a 20-dph larva exhibiting a weak
969 T_4 immunoreactivity within the colloid (D1 treatment); (e) and (f), thyroid follicles of 30-
970 dph larvae showing a moderate T_3 immunostaining (D1 treatment); (g), thyroid follicles
971 of 41-dph larvae showing moderate T_3 immunoreactivity (D1 treatment). Note the
972 increase of T_3 staining for the D3 treatment (h). Changes in the thyroid gland
973 development at 48 dph when comparing D1 with D4 treatments: note the decrease in
974 the number of follicles and the increase in their mean size, coupled with an increase of
975 T_4 staining intensity on *S. senegalensis* larvae from D4 treatment [(i) and (j), D1; (k)
976 and (l)]. Scale bars represent 100 μm.
977
978
979 Figure 7. Metamorphosis stages of Senegalese sole larvae fed graded levels of vitamin
980 A. Staging was established according to Fernández-Díaz et al. (2001). Different
981 indexed letters show significant differences among treatments (ANOVA, $P < 0.05$).
982
983
984 Figure 8. Incidence of skeletal deformities affecting the head, vertebral column, and tail
985 in Senegalese sole fed graded levels of vitamin A (a). Incidence of deformities
986 considering the number of abnormal skeletal elements per fish (b). Cranial deformities
987 in Senegalese sole fed the highest dose of VA showing the most affected skeletal
988 elements of the opercular complex (c). Different indexed letters show significant
989 differences among treatments (ANOVA, $P < 0.05$).

Figure 9. Incidence of deformities in prehaemal and haemal vertebrae along the vertebral axis in Senegalese sole larvae fed different levels of vitamin A. Feeding treatments: D1 (a), D2 (b), D3 (c), and D4 (d).

Figure 10. Incidence of skeletal deformities in the vertebral column of Senegalese sole fed graded levels of vitamin A. Total vertebral (prehaemal and haemal) deformities (a), deformed prehaemal (b) and haemal (c) centrums, abnormal neural (d) and haemal (e) spines, and parapophysis (f). Different indexed letters show significant differences among treatments (ANOVA, $P < 0.05$).

Figure 11. Examples of different typologies of skeletal deformities found in Senegalese sole under the present experimental conditions. (a) General view of a 30-dph metamorphic larva with a severe deformity in the vertebral column. (b) Torsion (T) of the first three prehaemal (cephalic) vertebrae resulting in deformed preopercular (Po), interopercular (Io) and ceratohyal (Ch). Note the space between the head and the abdominal region (arrow) as an indicator of the head's torsion. (c) Ectopical structure connecting neural spines from two adjacent haemal vertebrae (arrow). (d) Compression of centrums of haemal vertebrae and haemal vertebra with deformed haemal prezigapophysis (Hprz) and poszigapophysis (Hpz). (e) Fusion of haemal vertebrae numbers 43 and 44 with fusion of their respective haemal spines (asterisk). (f) Deformities affecting the caudal fin: deformed urostyle, fused hypurals 4-3 and 2-1, and fusion of hypural 1 with the modified haemal spine. (g) Compression of haemal vertebrae numbers 41-44 and disappearance of the intervertebral space among them. (h) Fusion of hypurals 1-5 and compression of haemal vertebrae (note the absence of intervertebral spaces among vertebral centrums). *Abbreviations*: Ep: epural; Hy:

1017 hypural; Mhs: modified haemal spine; Mns: modified neural spine; Phy: parahypural;
1018 Ur: urostile.
1019
1020 Figure 12. Incidence of deformities in the caudal fin complex in Senegalese sole fed
1021 graded levels of vitamin A. Percentage of specimens with at least one deformity in the
1022 caudal fin (a), parahypural (b), hypurals (c), epural (d), modified neural spine (e), and
1023 modified haemal spine (f). Different indexed letters show significant differences among
1024 treatments (ANOVA, $P < 0.05$).
1025
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Table 1

Table 1. Total lipid and retinoid content (retinyl palmitate, retinol and total VA) in experimental *Artemia* enriching emulsions. Total lipid content is expressed as % DW and retinoid content in emulsions is expressed as μg mg^{-1} DW. Different letters within the same column show significant differences between emulsions (ANOVA, $P < 0.05$).

Emulsion	Total lipids	Retinyl palmitate	Retinol	Total VA
D1	84.3 ± 2.94	1.23 ± 0.010 a	0.0051 ± 0.0005 a	1.32 ± 0.030 a
D2	81.7 ± 3.31	2.07 ± 0.440 ab	0.0057 ± 0.0003 ab	2.09 ± 0.123 b
D3	87.8 ± 6.25	4.47 ± 0.830 b	0.0079 ± 0.0005 b	4.50 ± 0.249 c
D4	82.7 ± 3.01	12.87 ± 0.198 c	0.013 ± 0.002 c	12.91 ± 0.059 d

Table 2

Table 2. Final larval size in standard length (SL) and dry weight (DW), and survival rate of Senegal sole larvae fed different levels of vitamin A. Values are mean ± standard deviation. Different letters within the same column show statistical significant differences.

Dietary treatment	SL (mm)	DW (mg)	Survival (%)
D1	13.35 ± 0.09 a	7.42 ± 0.40	47.1 ± 4.0
D2	11.91 ± 0.10 c	5.29 ± 0.30	45.6 ± 2.9
D3	12.40 ± 0.10 b	6.13 ± 0.29	41.6 ± 0.7
D4	11.84 ± 0.10 c	6.30 ± 0.40	41.3 ± 5.1

Table 3

Table 3. Differences in the number and size of thyriod follicles of Senegal sole larvae fed different levels of vitamina A. Different letters within the same age range (rows) show statistical significant differences among emulsions (ANOVA, $P < 0.05$).

Days post-hatching	Number of follicles				Size (mean ± SD) μm			
	D1	D2	D3	D4	D1	D2	D3	D4
10 dph	2	4	5	5	26.4 ± 3.21^{a}	26.0 ± 8.06^{a}	25.0 ± 8.05^{a}	25.4 ± 9.49^{a}
15 dph	4	5	6	6-7	25.7 ± 9.86^{a}	21.3 ± 3.40^{a}	26.1 ± 5.45^{a}	28.3 ± 8.19^{a}
20 dph	6	2-4	5	4-5	41.9 ± 1.58^{a}	39.1 ± 16.01^{a}	56.7 ± 5.79^{ab}	59.5 ± 2.78^{b}
30 dph	7	3-5	3-4	4-5	75.4 ± 8.55^{a}	95.1 ± 11.06^{a}	112.2 ± 2.23^{bc}	115.4 ± 3.34^{c}
41 dph	8	5	4-5	5	81.1 ± 2.13^{a}	114.1 ± 8.89^{b}	125.1 ± 9.12^{bc}	132.2 ± 8.23^{c}
48 dph	10-15	6	6-7	6	88.2 ± 5.24^{a}	124.0 ± 1.33^{b}	131.1 ± 5.56^{bc}	135.1 ± 6.69^{c}

Table 4

Table 4. Semiquantitative assessment of thyroid hormones content by using immunohistochemical approaches. Asterisks indicate reaction colour intensities: */- weak; * moderate; ** intense; ***very intense.

Days post-hatching	T_3 - immunoreactivity				T_4 - immunoreactivity			
	D1	D2	D3	D4	D1	D2	D3	D4
10 dph	*/-	*	*	*	*	*	*	*
15 dph	*/-	*/-	*	*	*	*	*	*
20 dph	*	*	*	*	*	*	*	*
30 dph	*	*	**	*	*	*	*	**
41 dph	*	*	**	**	*	*	*	***
48 dph	*	*	**	***	*	*	**	***

Figure 1

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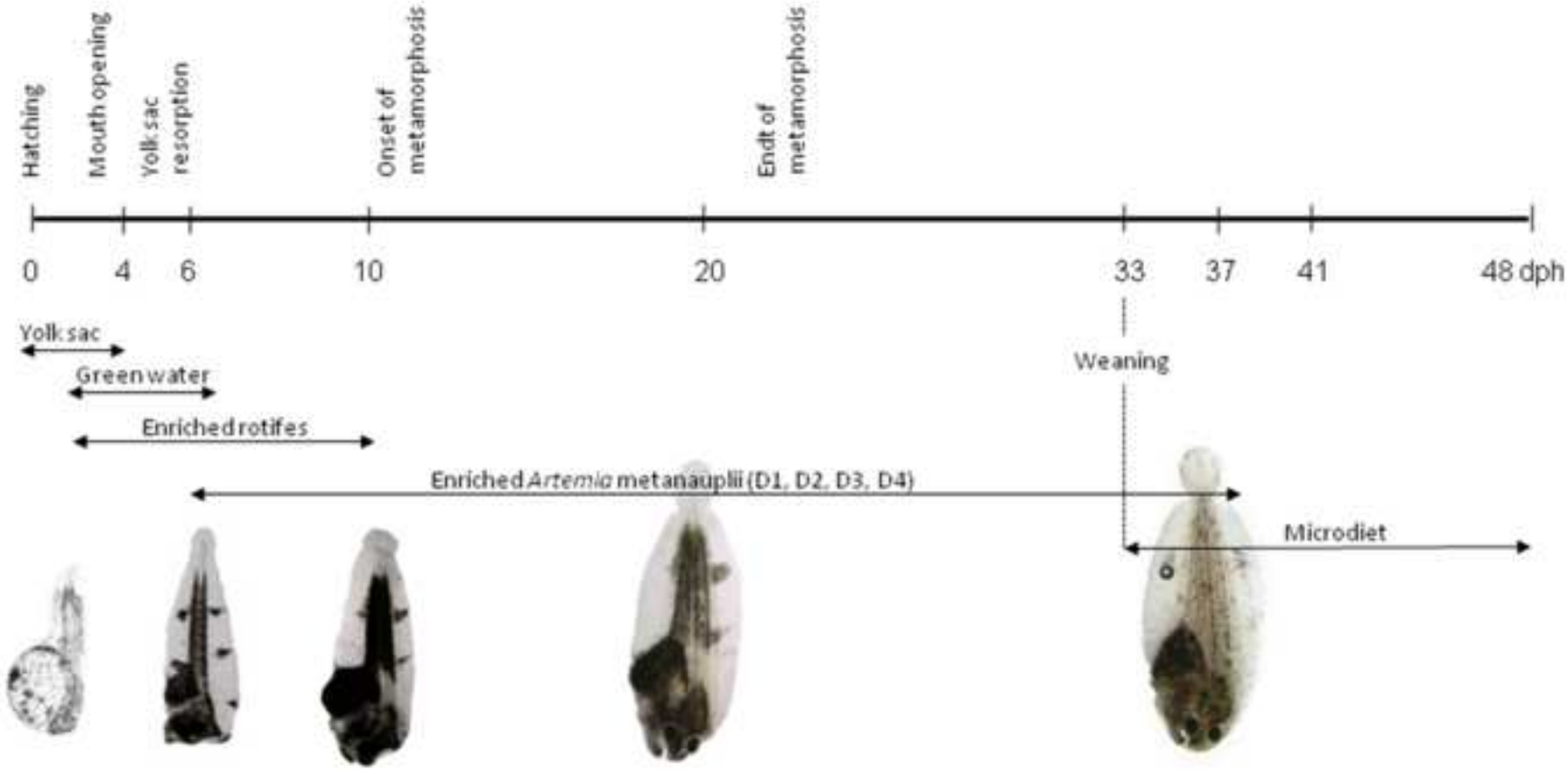

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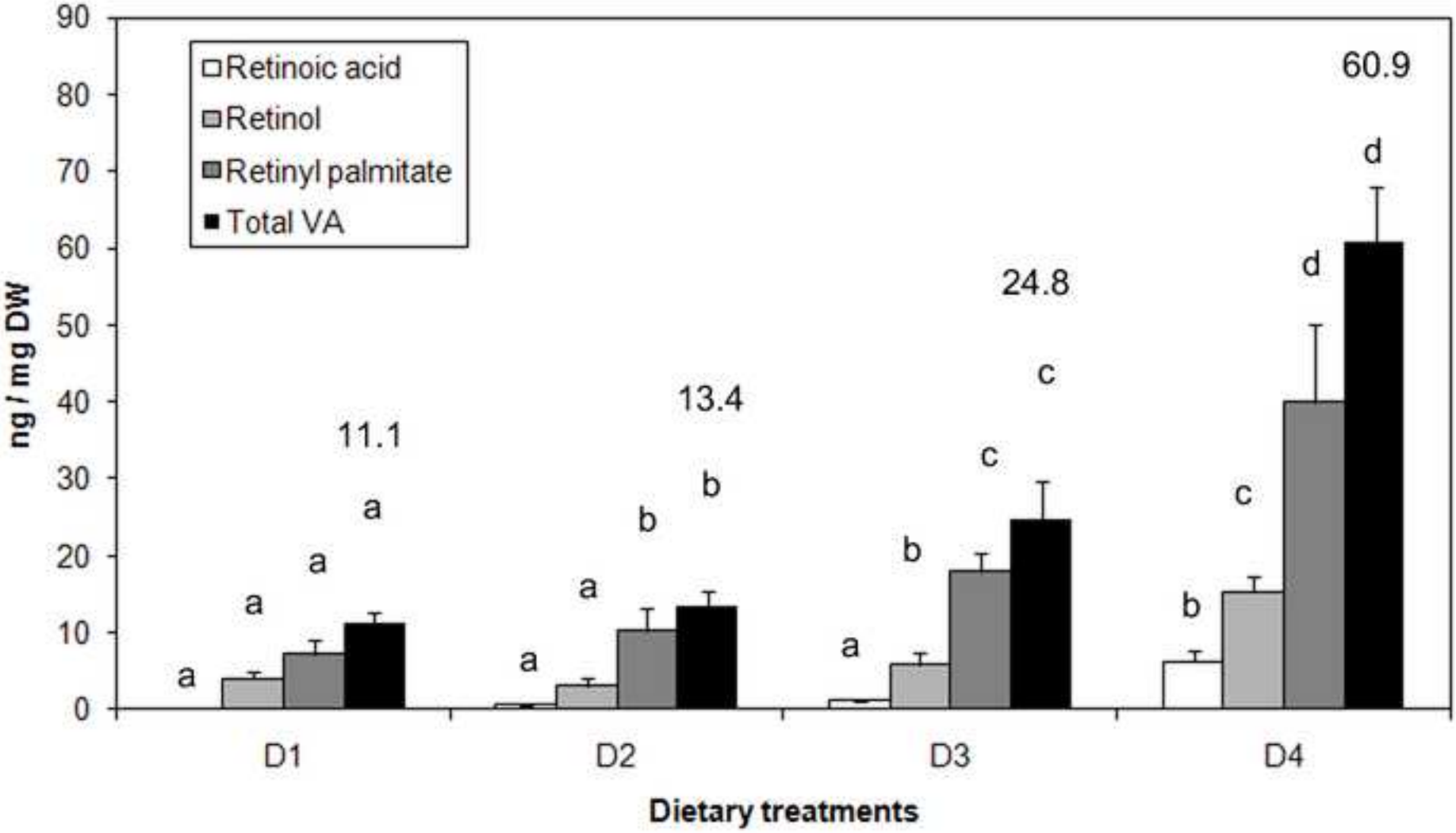

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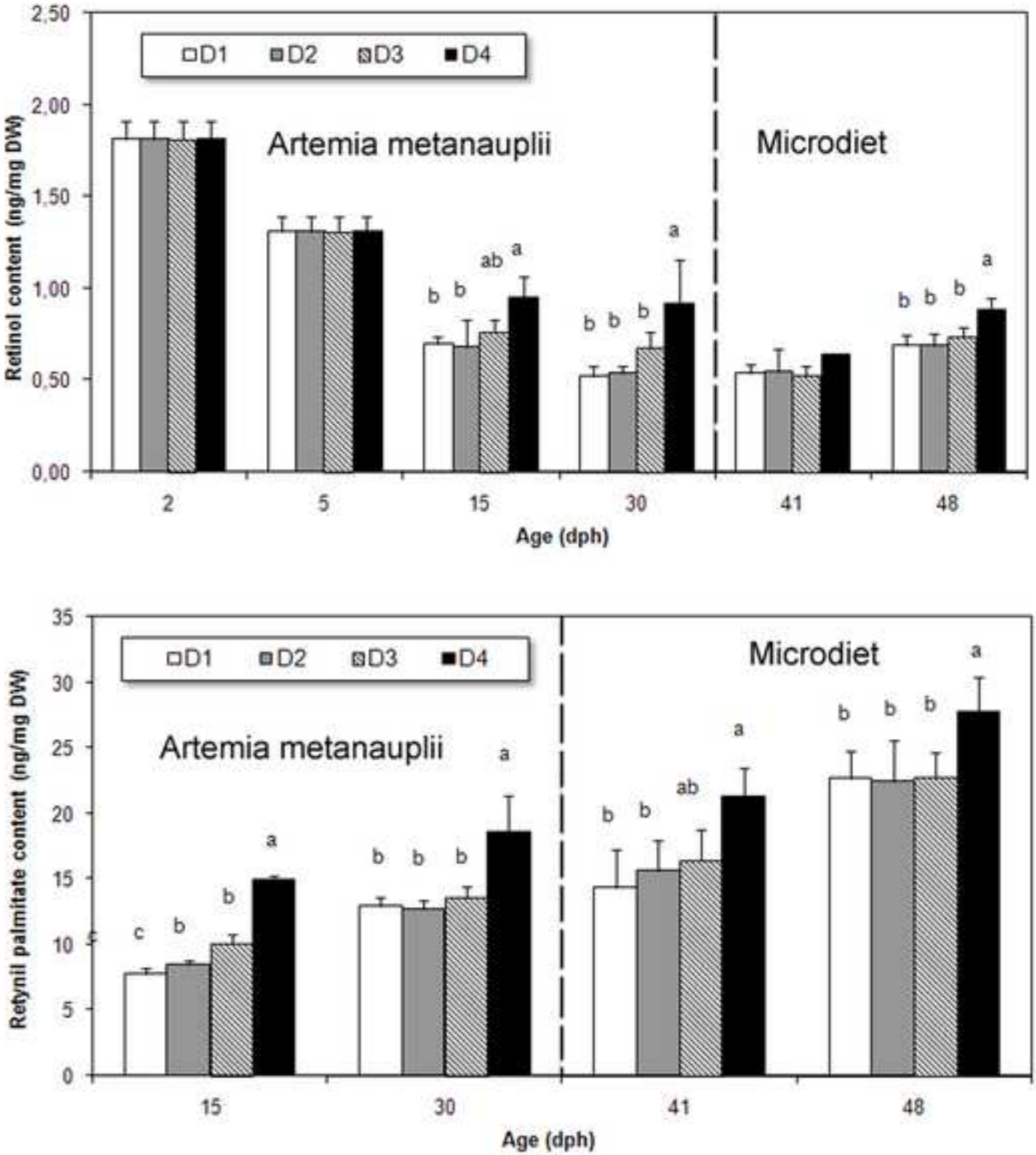

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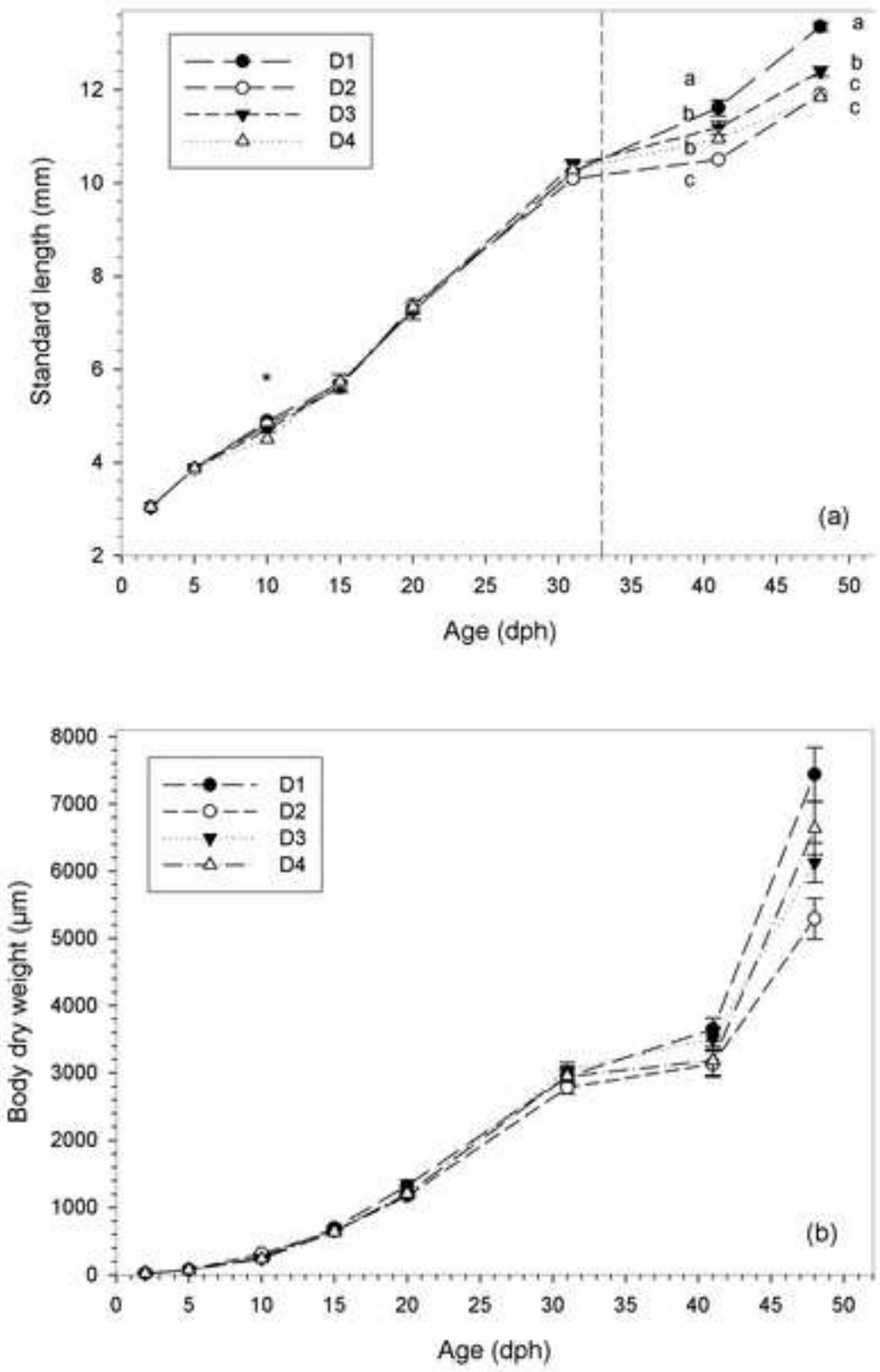

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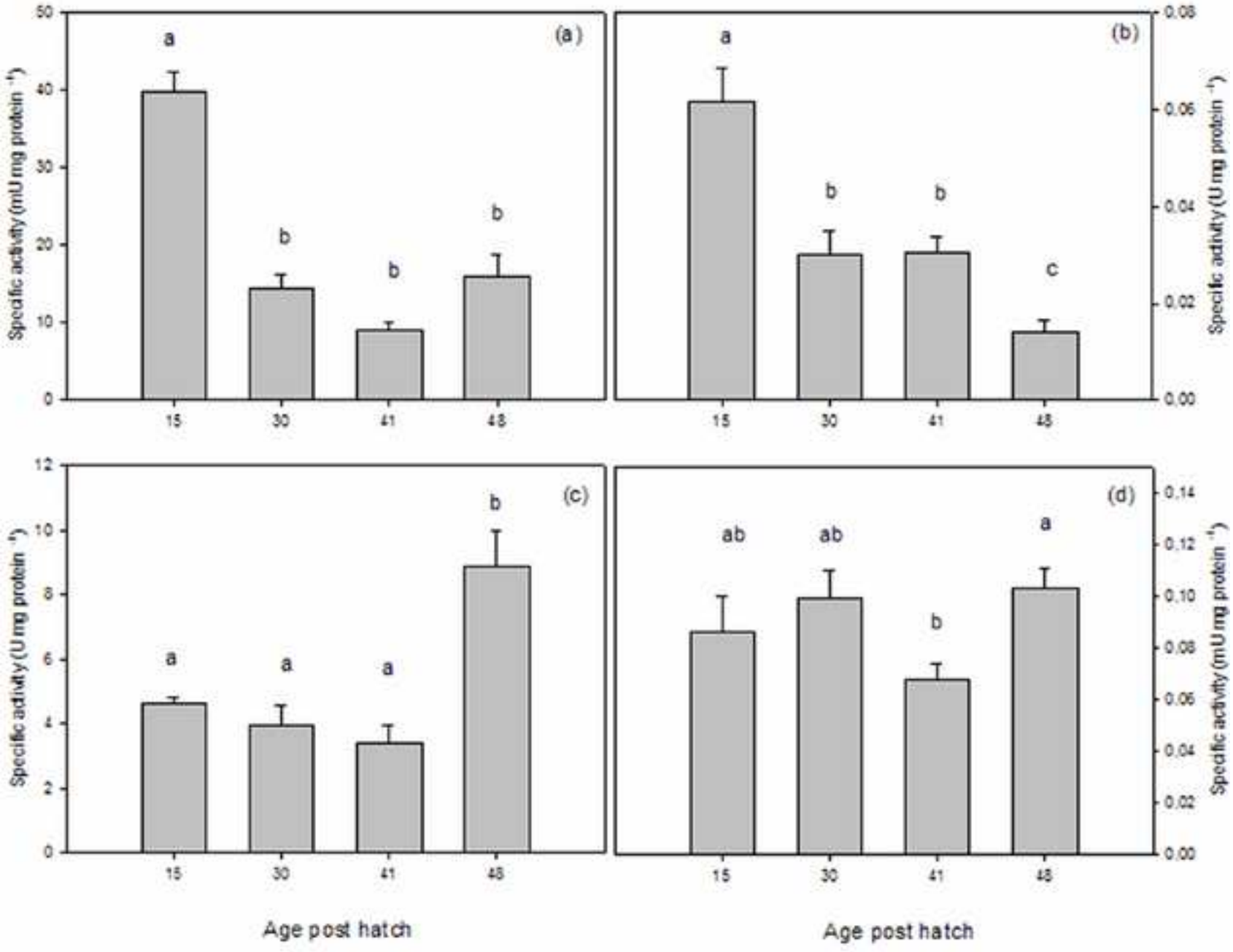

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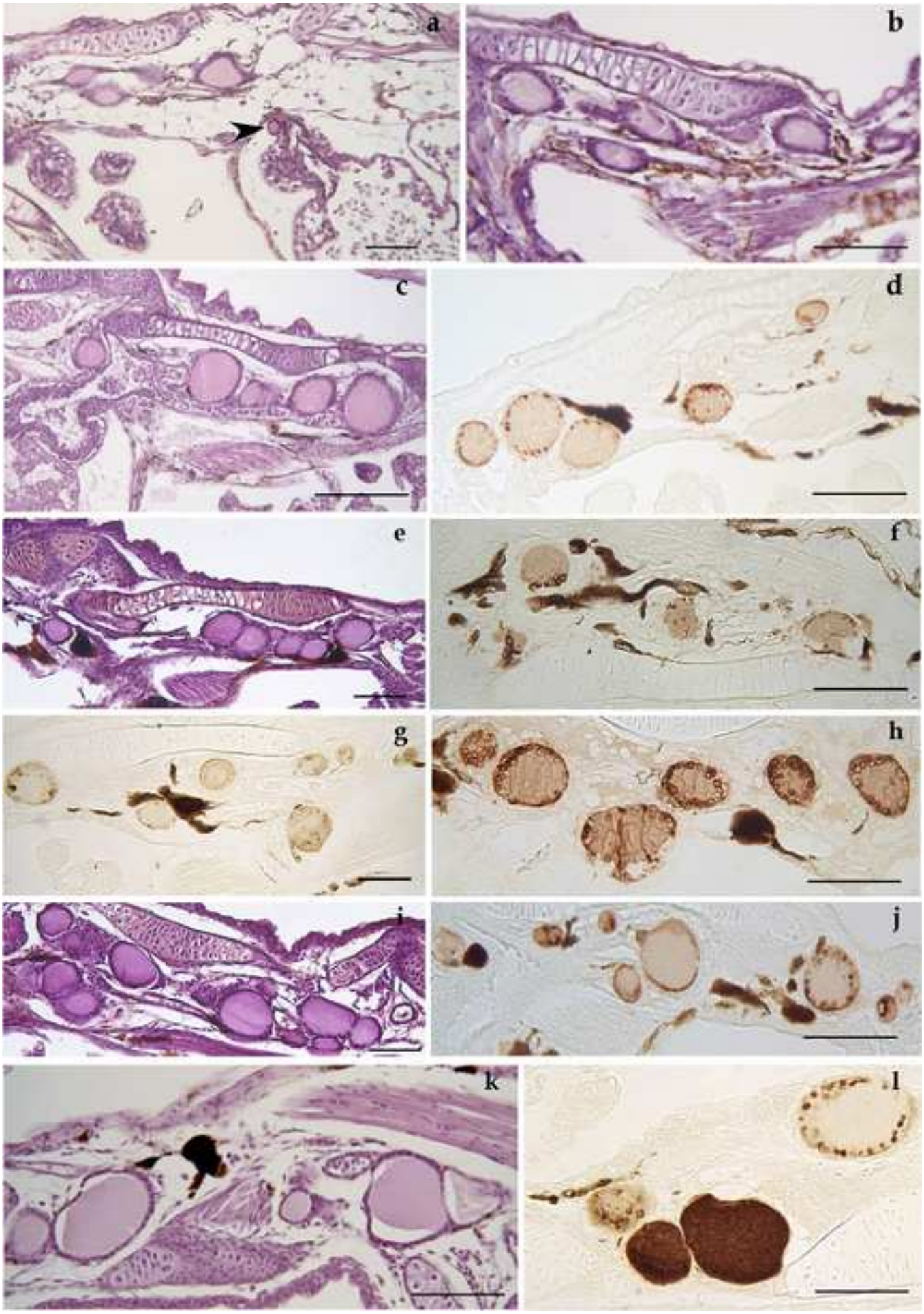

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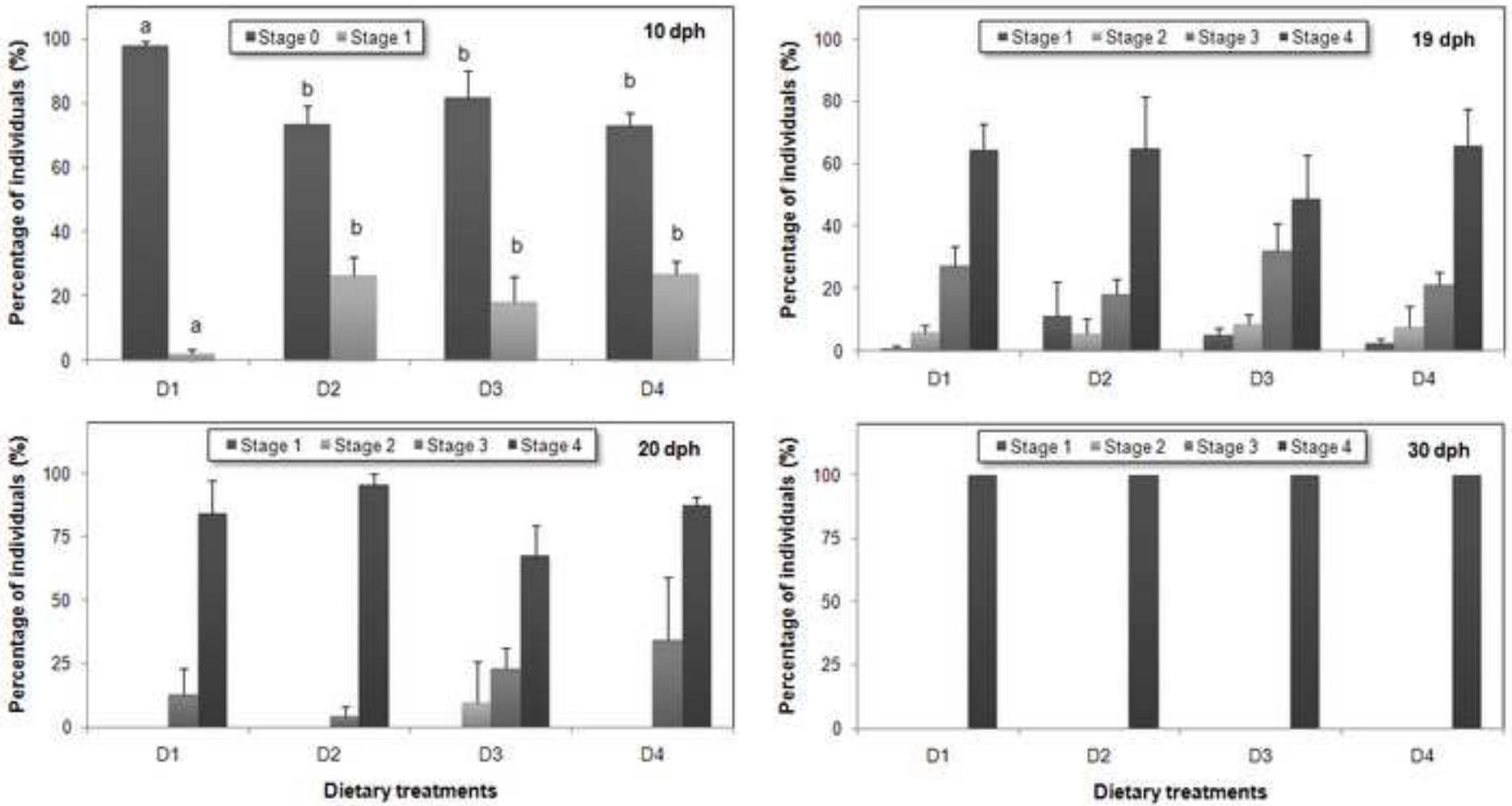

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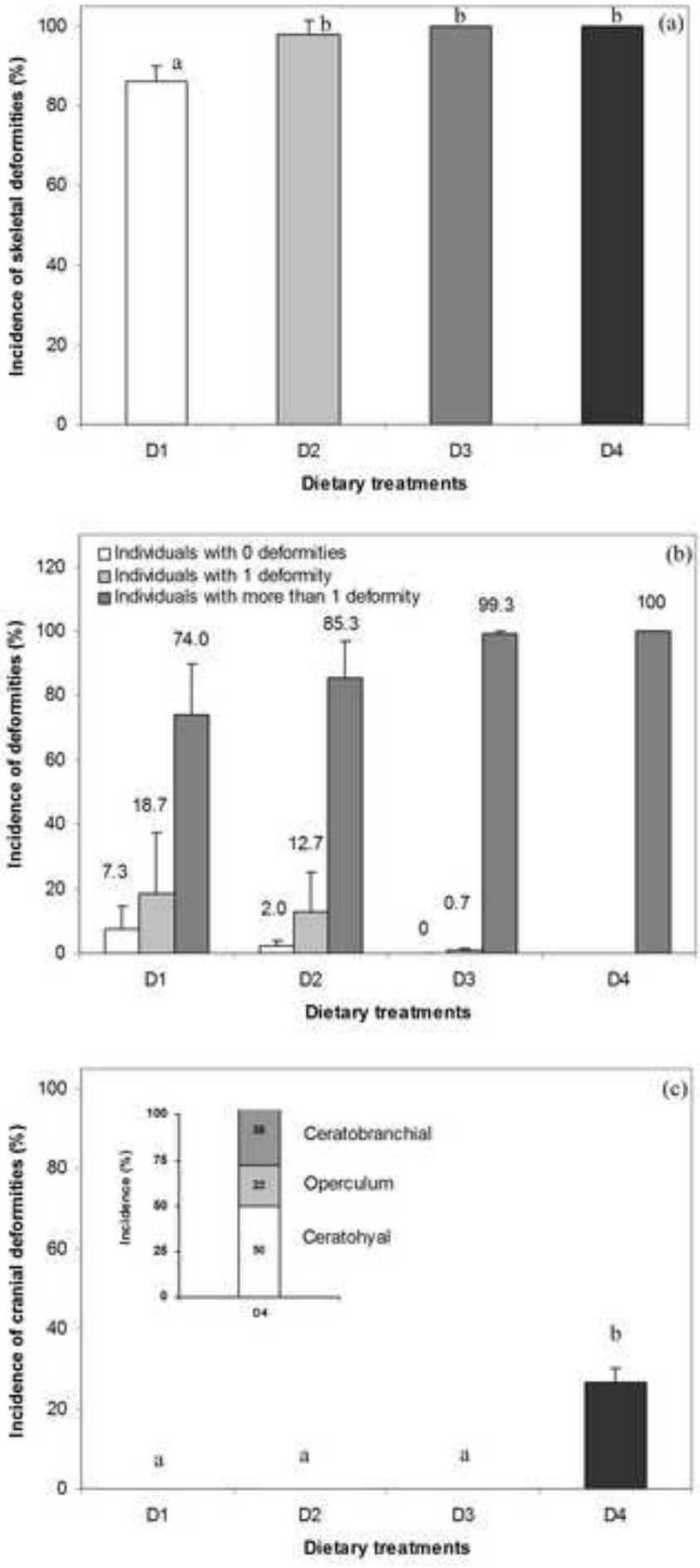

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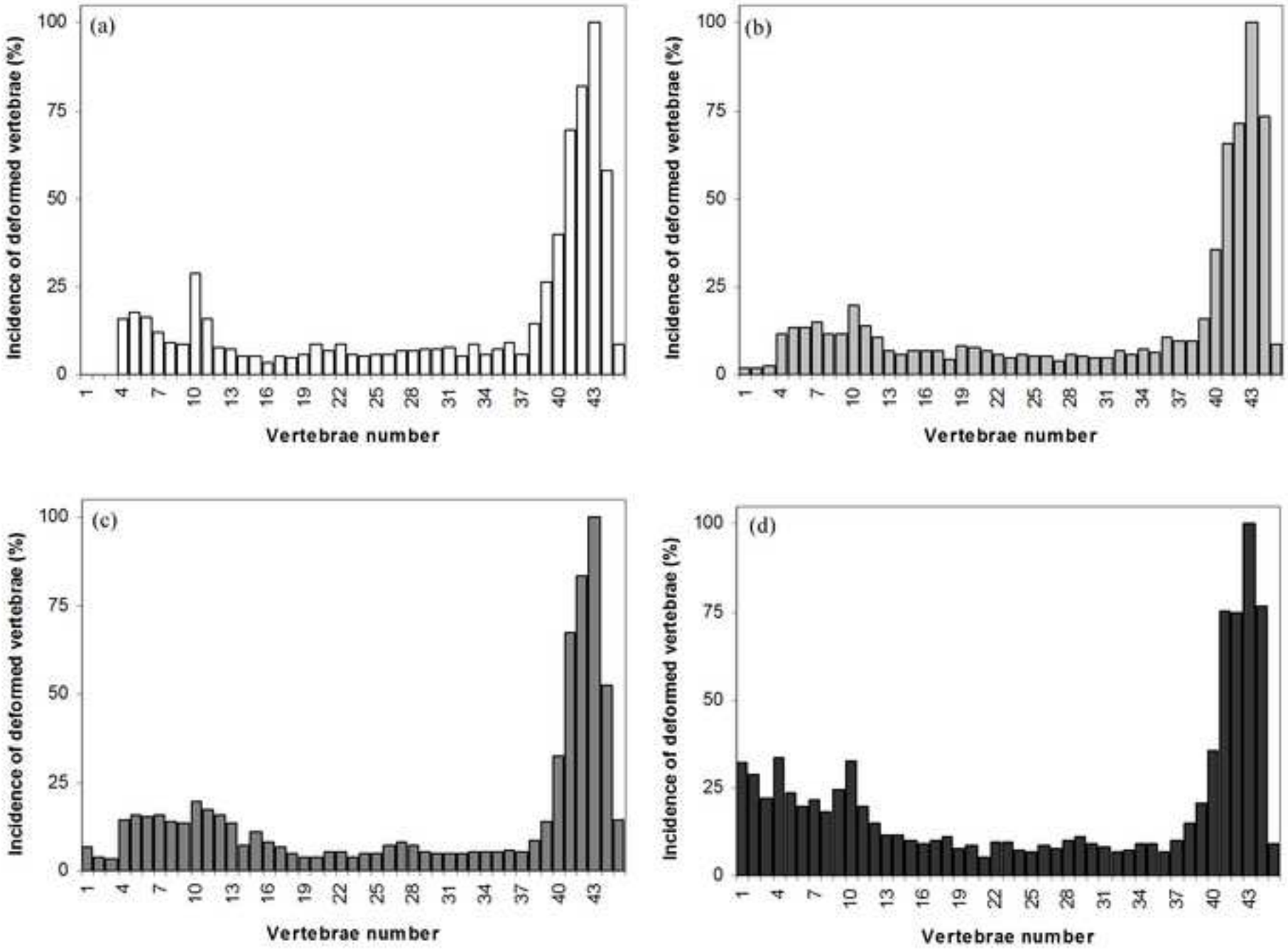

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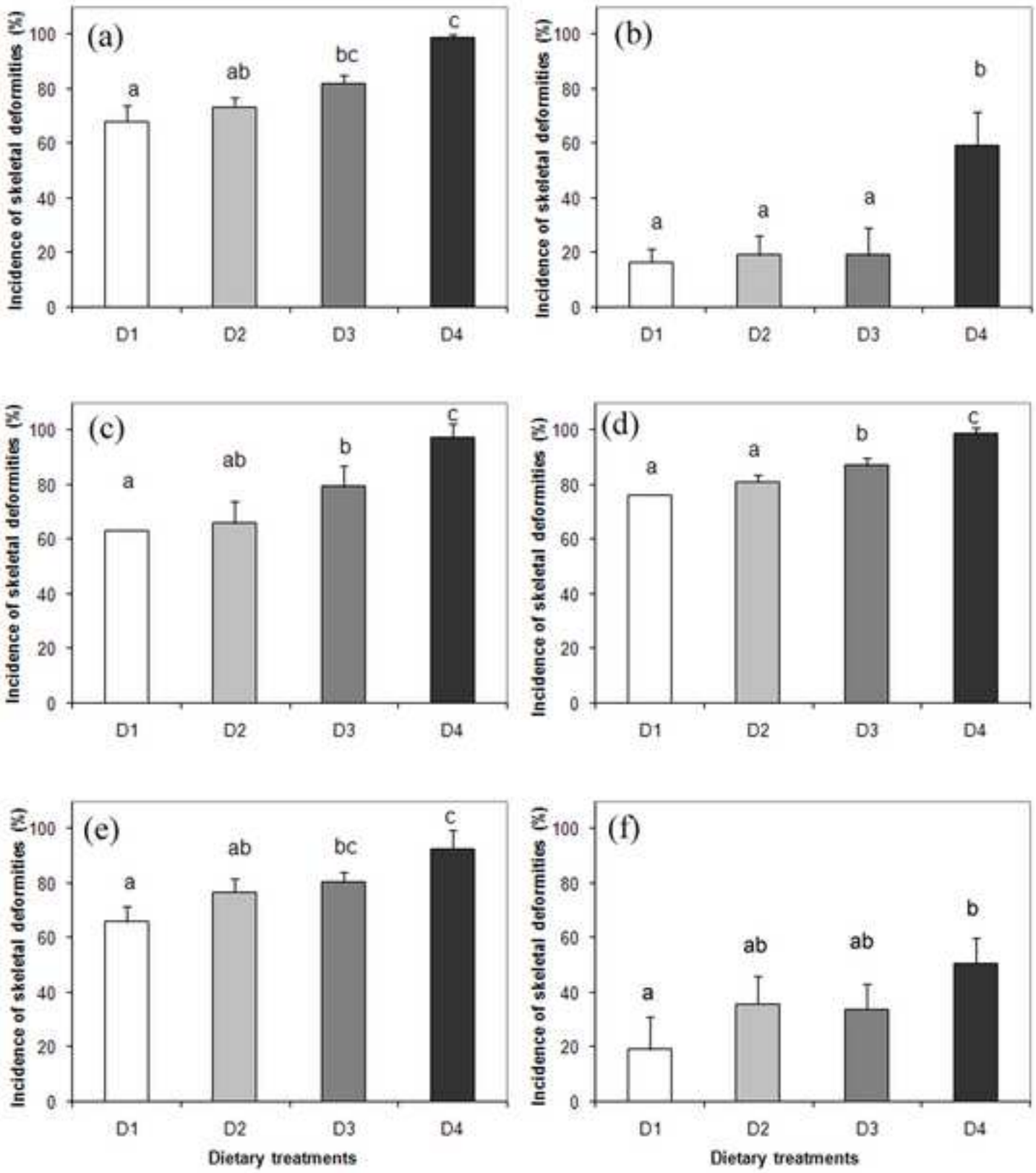

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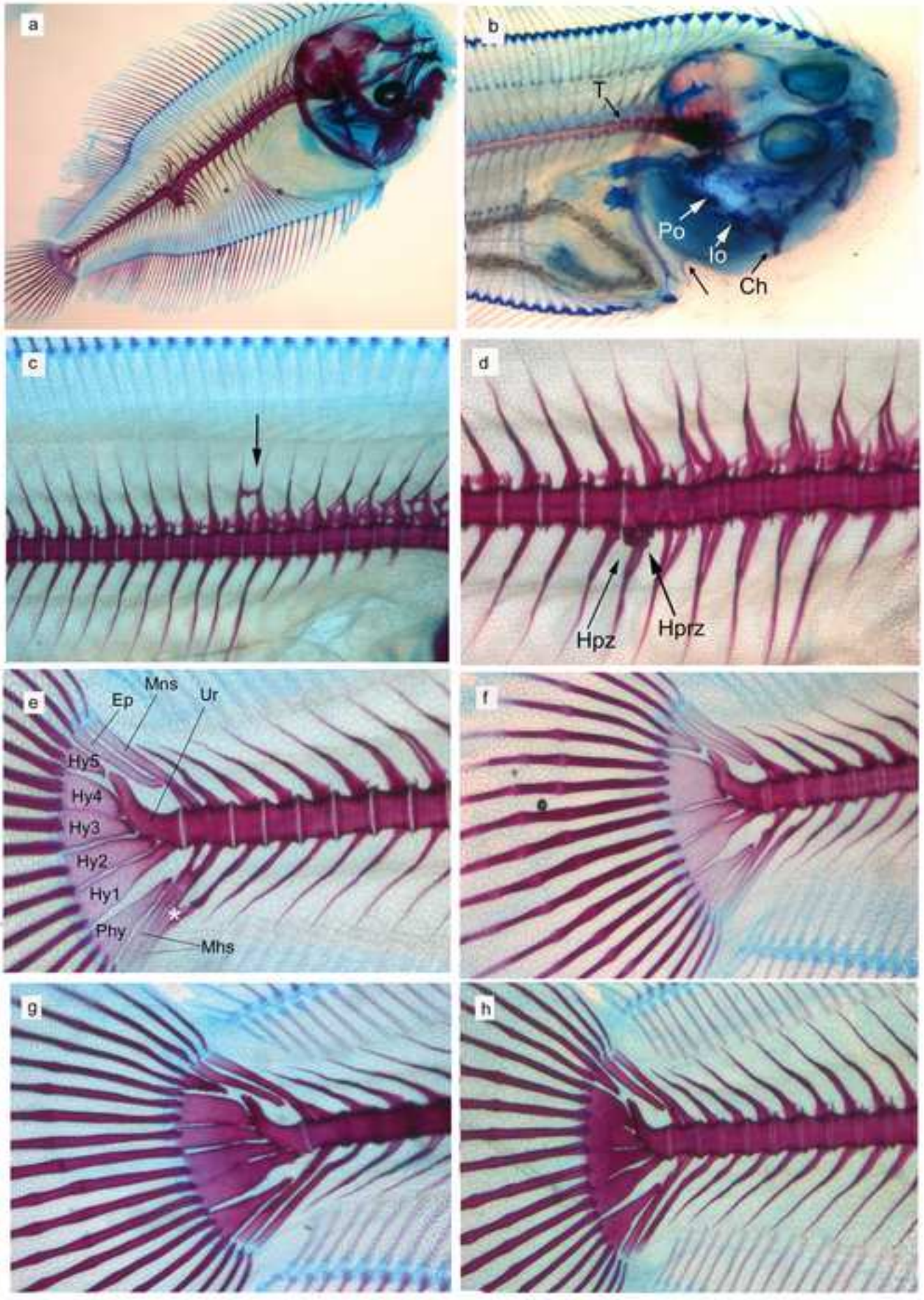

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